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14. ABSTRACT

The aim of this project is to dissect the role of Tbx2 in prostate cancer progression. In the present study we have found that by reducing the expression of Tbx2 in prostate cancer cell lines reduces proliferation in vitro. Further, utilizing tissue recombination techniques we found that the grafts obtained from NeoTag-2 mouse prostate cancer cells containing the dominant negative Tbx2 construct are more differentiated as compared to controls. These findings suggest that inducing senescence by down-regulating Tbx2 may reduce tumorigenesis and hence Tbx2 plays an important role in prostate cancer progression.

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a) Generate stable clones of prostate cancer cells in which Tbx2 is over-expressed or down-regulated. (Months 12-16)
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b) Use *in vivo* grafts to delineate the effect of modulating Tbx2 levels on the resistance to chemotherapeutic drugs. (Months 28-36)

Introduction:

Prostate cancer is the second leading cause of cancer deaths in men. Initially all prostate tumors respond to anti-androgen therapy but in almost all cases, the tumors become androgen insensitive. The majority of prostate tumors metastasize to the bone and bone metastases are the primary cause of death in prostate cancer cases. One of the ways to treat metastases is through chemotherapy with drugs such as doxorubicin. However tumors develop resistance to chemotherapeutic drugs and escape therapy. Senescence has in recent years been identified as a mechanism that can influence cancer therapy. As opposed to apoptosis or cell death senescence can be induced by relatively low drug concentrations thus circumventing the problem of drug resistance and or drug toxicity issues in tumors. Thus identifying mechanisms by which senescence can be induced in prostate cancer cells can be a potential approach to the treatment of prostate cancer. We found (in the preliminary data submitted in the original DOD grant application) that Tbx2, an anti-senescence transcription factor is over-expressed in a subset of human and mouse prostate cancer cell lines. Further we showed (in the original grant) that Tbx2 expression is regulated by androgens both in LNCaP cells and in the mouse prostate *in vivo.* Based on this preliminary data, Aim 1 proposes to study the androgen regulation of Tbx2. In Aim 2, we have proposed to study the *in vivo* effects of Tbx2 manipulation in prostate cancer cells using a tissue recombination approach to simulate the prostate microenvironment and also to study these Tbx2 manipulated prostate cancer cells in a bone microenvironment using bone grafts. Aim 3 proposes to study the role of Tbx2 in the resistance to pharmacological agents like doxorubicin in prostate cancer cells. Overall, the grant proposes to study the role of Tbx2 in prostate cancer progression.

Body:

Task 1: Characterize androgen regulation of Tbx2. (Months 1-11)

Since we showed in the preliminary data that Tbx2 expression is induced by the addition of androgens both in LNCaP cells and in mouse prostate *in vivo,* we wanted to determine if androgens directly regulate Tbx2 expression. To test the possibility if the androgen receptor (AR) binds and regulates Tbx2 expression, we utilized a 960 base pair Tbx2 promoter-luciferase construct, transfected in LNCaP cells and performed a luciferase assay. Since BMP2 and retinoic acid are also known to regulate Tbx2 expression, we included BMP2 and retinoic acid treatments in addition to androgens (DHT). Following serum starvation, we treated LNCaP cells with either DHT, BMP2, retinoic acid or in combinations. **(Fig. 1)** Neither individual treatments nor combinations of the three produced any appreciable luciferase activity. In contrast, the positive control for the luciferase experiment, the ARR2PB luciferase construct gave a robust activity with the addition of DHT. It is possible that the GRE / ARE sequence in the 960 bp Tbx2

promoter is not the correct hormone response element or that it functions in a cooperative manner with additional AREs that are further upstream of the 960 bp promoter. Therefore we are in the process of PCR cloning a larger Tbx2 construct (up to 4000 base pairs) from genomic clones and test the larger promoter-luciferase tagged construct for androgen regulation.

Task 2: Determine the effect of signaling between androgen receptor and BMP2 on Tbx2 expression in the formation of prostate tumors and growth in bone microenvironment (Months 12-24):

Since it is previously known that BMP2 induces Tbx2 expression in other developmental model systems, we wanted to test if BMP2 can stimulate Tbx2 expression in prostate cancer cells. Following serum starvation for 48 hours, we treated LAPC4 cells, an androgen dependent human prostate cancer cell line with BMP2 at a concentration of 50 ng/ml. BMP2 induced Tbx2 expression robustly after 24 hours of treatment and remained induced in the 48 and 72 hour time points. **(Fig. 2)** Tbx2 is known to inhibit p16 arf expression and we observed inhibited p16 arf expression at 24 and 48 hour time points. **(Fig. 2)** The increased p16 arf expression at the 72 hour time point is not clear at this time. Though Tbx2 is also known to inhibit p21 expression, we observed that p21 expression is also induced after 24 hours. **(Fig. 2)** Since BMP2 is known to signal through Wnts, we checked the expression of Wnt genes following BMP2 addition to LAPC4 cells. Of all the canonical and non-canonical Wnts we examined by RT-PCR, we observed that Wnt 3a, a canonical Wnt, is induced after 24 hours following the addition of BMP2. **(Fig. 2)** The induction of Tbx2 and Wnt 3a following BMP2 addition has interesting implications from the bone metastasis point of view. LAPC4 cells are known to form osteoblastic lesions in the bone microenvironment and it is known that BMP2 and Wnt signaling pathways play a crucial role in the formation of bone lesions. We are planning to perform the bone graft experiment soon as proposed in Aim 2. It will be very interesting to see what kind of lesions will form when prostate cancer cells in which Tbx2 expression is downregulated with the help of the dominant negative Tbx2 construct are placed in the bone microenvironment as compared with the empty vector controls.

Task 2a: Generation of stable clones of prostate cancer cells in which Tbx2 is down-regulated (Months 12-16): We chose two androgen independent human prostate cancer cell lines DU145 and PC3 to knock-down Tbx2 expression with the help of a Tbx2 dominant negative construct. We also chose the NeoTag-2 cells (a cell line generated from the LPB Tag 12T-7 mouse model of prostate adenocarcinoma) to knock-down Tbx2 expression. The NeoTag-2 cells are androgen dependent. In addition, we used the LAPC4 cells to introduce the dominant negative construct. The NeoTag-2 cells when recombined with rat UGM (to induce prostate development) and placed in the kidney form foci of adenocarcinoma. Therefore it is interesting to see what effect knocking down of Tbx2 has on the adenocarcinoma morphology formed by Neotag-2 cells recombined with UGM. We characterized all the four prostate cancer cell lines stably infected with the Tbx2 dominant negative construct for HA tag (the Tbx2 DN construct contains an HA tag), p21 or p19 arf expression. As expected, all four cell lines having dominant

negative vector showed the expression of HA Tag. **(Fig. 3)** All the three human cell lines LAPC4, PC3 and DU145 showed enhanced expression of p21 expression as expected. **(Fig. 3)** However the NeoTag-2 cells showed decreased expression of p19 arf contrary to expectations. **(Fig. 3)**

Prostate cancer cells with Tbx2 DN vector display decreased proliferation potential *in vitro*: (Fig. 4)

Task 2b: Tissue recombination of prostate cancer cells carrying the Tbx2 DN vector (Months 16-24) : We utilized the technique of tissue recombination to delineate the contribution of Tbx2 in prostate carcinogenesis *in vivo.* The rat urogenital mesenchyme (UGM) provides an inductive signal to the prostate epithelial cells for the formation of prostate. We used 300k UGM cells to recombine with 100k epithelial cells carrying either the Tbx2 DN vector or the empty vector (EV). The cells were mixed and the cell pellet was placed beneath the kidney capsule of the nude mouse. Eight weeks later, the grafts were harvested and examined by H & E staining. The NeoTag-2 is a mouse prostate cancer cell line that was developed from the 12T-7 mouse model of prostate cancer. These cells when recombined alone with rat UGM develop foci of adenocarcinoma. Interestingly, the Tbx2 DN Neotag-2 cells formed differentiated glands in addition to foci of adenocarcinoma whereas the NeoTag-2 cells carrying the empty vector (EV) predominantly formed adenocarcinoma. **(Fig. 5)** This fits with the data that NeoTag-2 cells infected with the Tbx2 DN vector show decreased proliferation potential in vitro. We did not observe any difference in graft volume for the NeoTag-2 grafts. In contrast, LAPC4 cells with Tbx2 DN construct formed grafts that were larger as compared to EV LAPC4 grafts. **(Fig. 6)** The LAPC4 cells do not form any appreciable glandular histology unlike the NeoTag cells. **(Fig. 7)** We are now in the process of characterizing these NeoTag-2 and LAPC4 grafts with various immunohistochemical markers for senescence like HA tag, p21, p19 arf and SA beta-galactosidase (a marker for senescent cells). The fact that the LAPC4 graft size is larger is puzzling. But given that the induction of senescence has been reported to induce tumorigenesis in certain environments, this may be a plausible explanation for the larger graft sizes. The Tbx2 DN LAPC4 grafts histologically show a lot of lymphocyte infiltration suggesting an inflammatory process. This may account for the larger graft size.

Task 3: Determine the role of Tbx2 signaling in resistance to pharmacological agents in human prostate cancer cells (Months 24-36):

We have not addressed this task partly or fully as yet.

List of Figures

Fig. 1

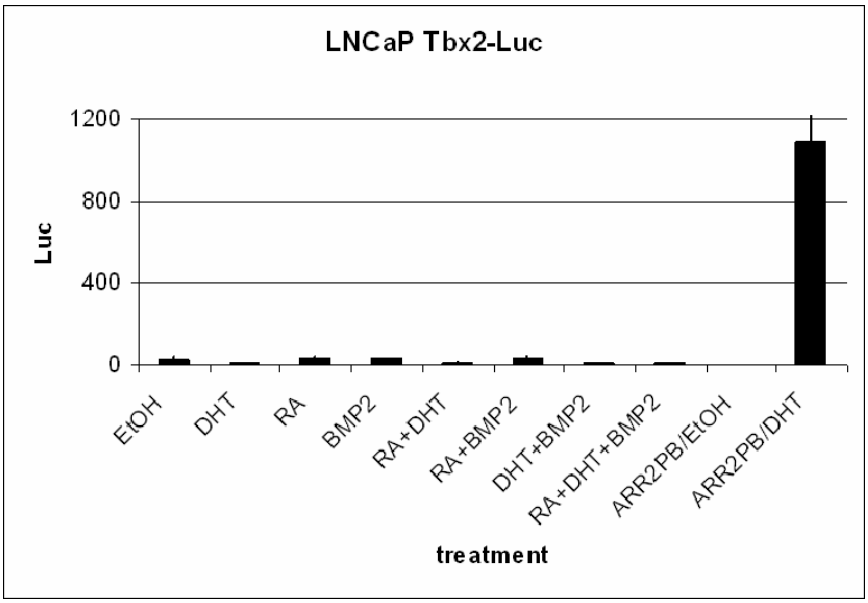


Fig. 2

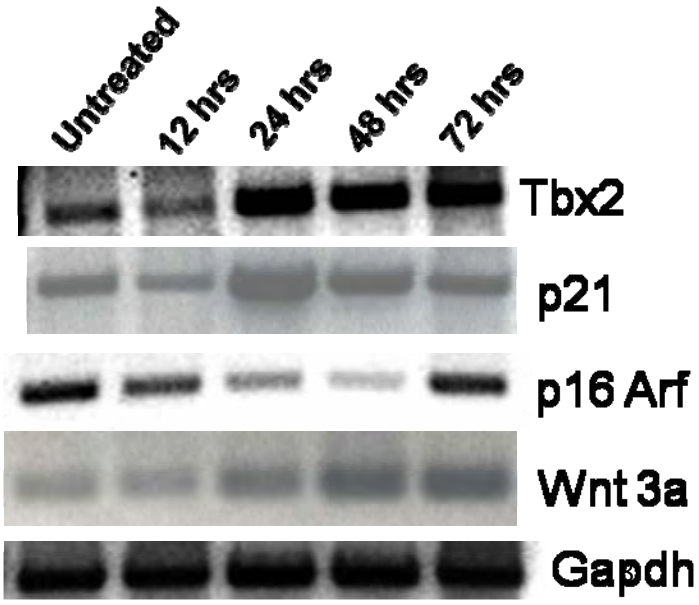


Fig. 3

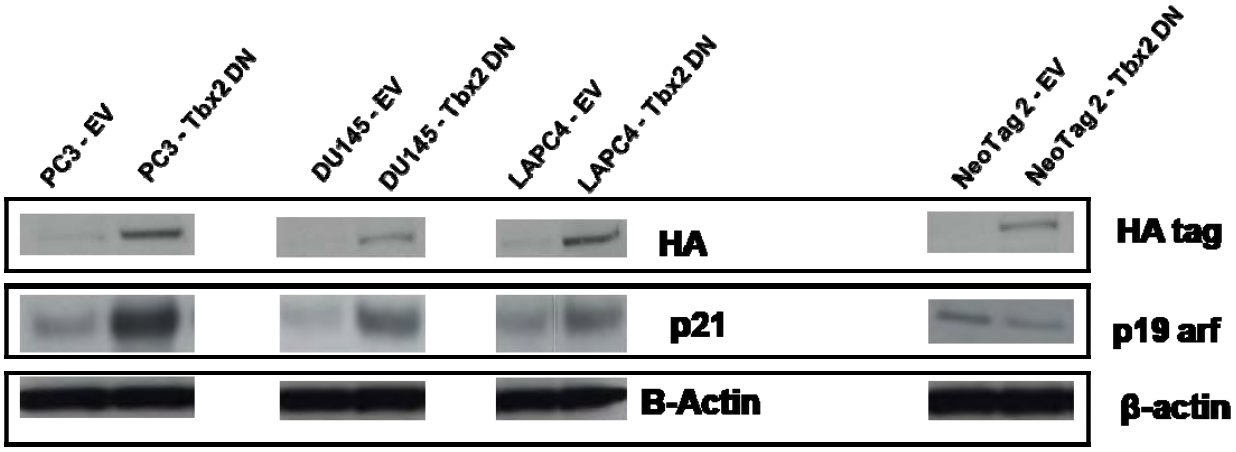

PC3 - EV
PC3 - Tbx2 DN
DU145 - EV
DU145 - Tbx2 DN
LAPC4 - EV
LAPC4 - Tbx2 DN
NeoTag2 - EV
NeoTag2 - Tbx2 DN
HA
HA tag
p21
p19 arf
B-Actin
β-actin

Fig. 4

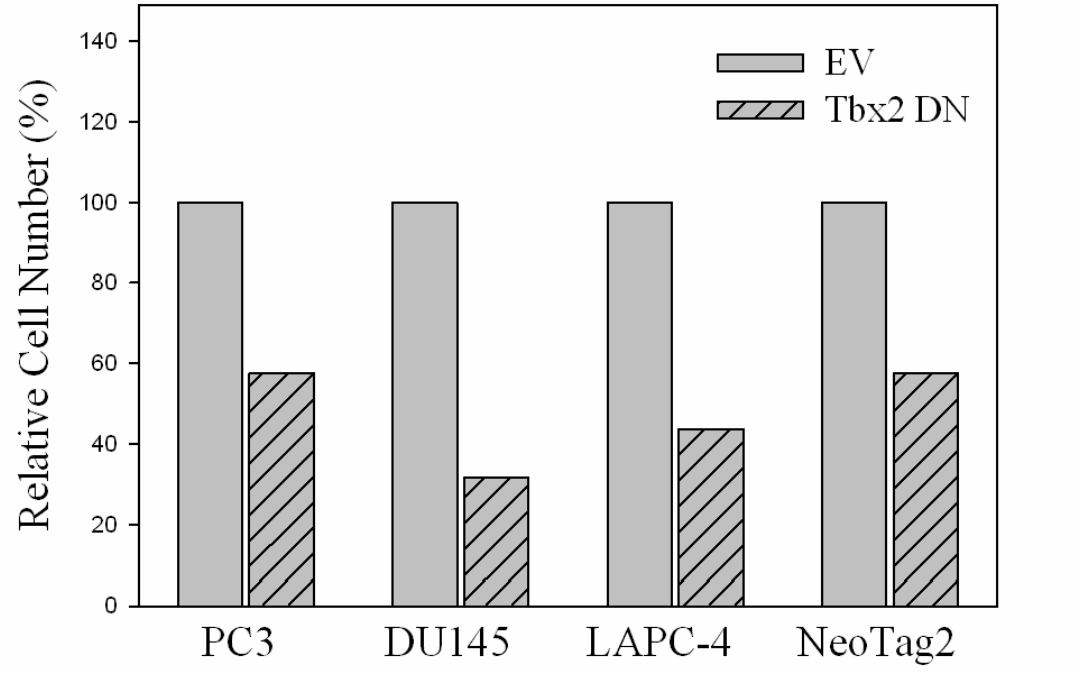

Relative Cell Number (%)
140
120
100
80
60
40
20
0
EV
Tbx2 DN
PC3
DU145
LAPC-4
NeoTag2

Fig. 5

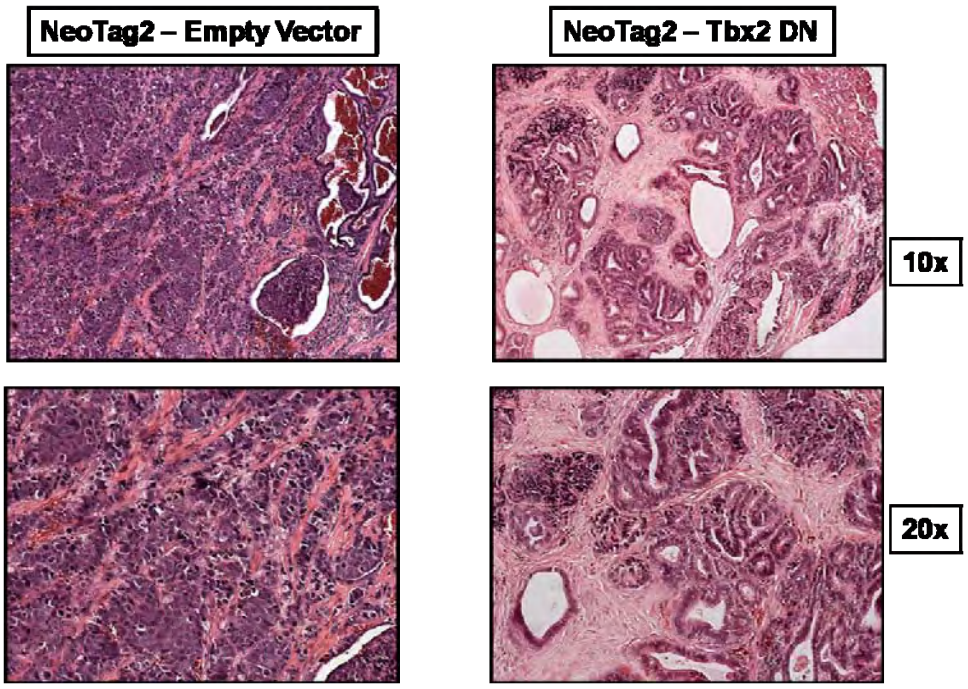

NeoTag2 – Empty Vector
NeoTag2 – Tbx2 DN
10x
20x

Fig 6.

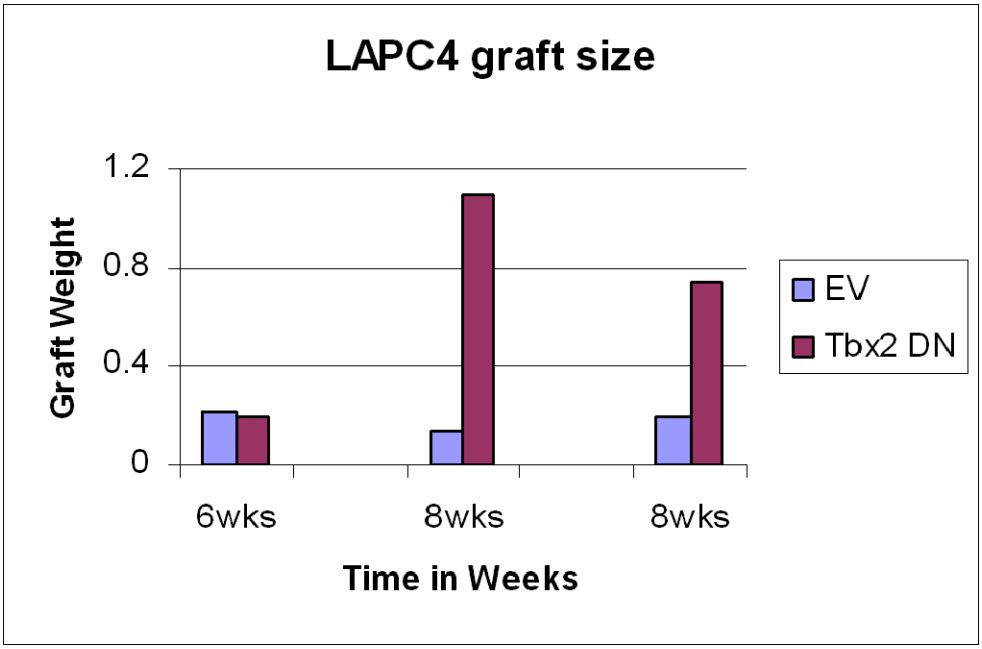
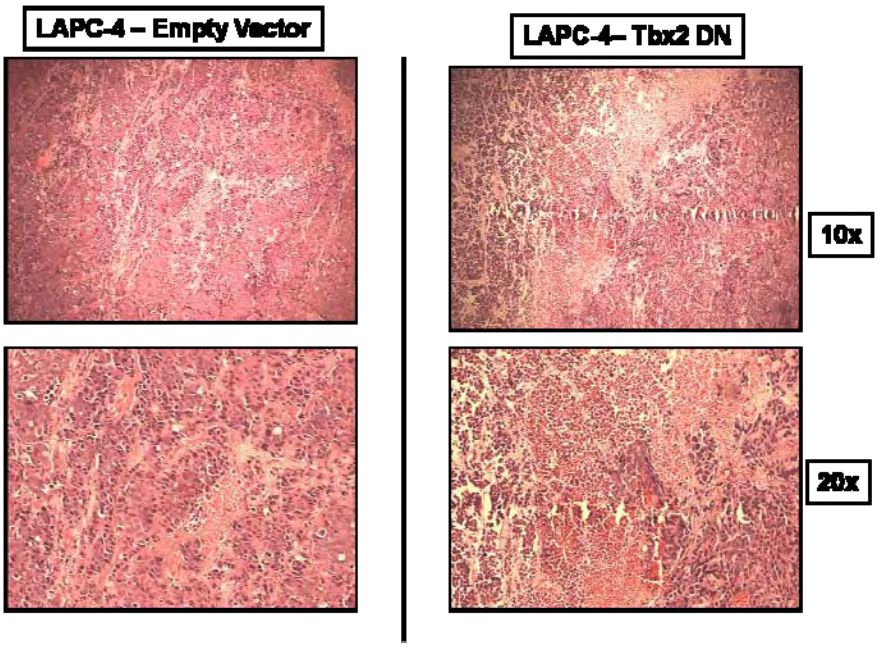

LAPC4 graft size
Graft Weight
1.2
0.8
0.4
0
6wks
8wks
8wks
Time in Weeks
EV
Tbx2 DN

Fig. 7



Key Research Accomplishments:

1. The androgen receptor (AR) does not bind to the 960 base pair Tbx2 promoter fragment we have used.

2. BMP2 treatment of LAPC4 cells induces Tbx2 and Wnt 3a expression after 24 hours of treatment.

3. Prostate cancer cell lines infected with the Tbx2 dominant negative construct show decreased cell proliferation *in vitro*.

4. NeoTag-2 mouse prostate cancer cells infected with Tbx2 DN construct when recombined with rat UGM and placed under the kidney capsule of mice form tumors that are more differentiated in histology as compared with NeoTag-2 cells infected with the empty vector (EV).

5. LAPC4 human prostate cancer cells infected with Tbx2 DN construct when recombined with rat UGM and placed under the kidney capsule of mice form tumors that are larger as compared with the empty vector control.

Reportable Outcomes:

Cell Lines and tissue grafts created

1. Development of NeoTag-2-pbabe puro-Tbx2 DN and NeoTag-2-pbabe puro-EV cell lines.
2. Development of tissue recombination grafts using NeoTag-2-pbabe puro-Tbx2 DN and NeoTag-2-pbabe puro-EV cell lines.
3. Development of LAPC4-pbabe puro-Tbx2 DN and LAPC4-pbabe puro-EV cell lines.
4. Development of tissue recombination grafts using LAPC4-pbabe puro-Tbx2 DN and LAPC4-pbabe puro-EV cell lines.

Conclusion: It is widely known that cellular senescence plays an important role in cancer progression. Reports have suggested that senescence can either decelerate the process of tumorigenesis or promote tumorigenesis depending on the tumor microenvironment. The NeoTag-2 cells applied to tissue recombination model provides a good tool to study prostate cancer progression because of the evident glandular morphology of the tumor akin to prostate tumors. We have observed that reducing the expression of Tbx2 in NeoTag-2 cells leads to the formation of a more differentiated tumor morphology. Conversely, reducing the expression of Tbx2 in LAPC4 cells resulted in bigger tumors. We are now in the process of confirming our results by performing more tissue recombination grafts and characterize these tumors immunohistochemically with markers of senescence and proliferation and apoptosis.

So what section: By regulating Tbx2 expression in prostate tumors, it may be possible to induce senescence and hence control the tumor growth rate and/or push the tumor cells towards differentiation.

References:

N/A

DN: Dominant Negative
BMP2: Bone Morphogenic Protein 2
DHT: Dihydrotestosterone
GRE: Glucocorticoid Response Element
ARE: Androgen response Element